IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Eiji MORI *et al.*	**Filed via EFS Web March 26, 2008**
Title:	ANTI-TRAIL-R ANTIBODY	
Appl. No.:	10/721,763	
International Filing Date:	5/17/2002	
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Examiner:	Claire M. Kaufman	
Art Unit:	1646	
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DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Kazuhiro Motoki, declare that

1. I am a citizen of Japan and am employed as a Senior Scientist in the Discovery Research Laboratories of KIRIN PHARMA COMPANY, LTD., which is located at 3 Miyahara-cho, Takasaki-shi, Gunma, 370-1295, Japan. I graduated from Graduate School of Pharmaceutical Sciences, Chiba University with a master's degree in 1991, and obtained a PhD at Niigata University in 1996. At KIRIN PHARMA COMPANY, I studied the tumor immunology for more than 15 years. In detailed, I studied the antitumor efficacy and mechanisms of action of novel immunomodulator, α-galactosylceramide, from 1992 through 2000. For last 7 years, I studied the antitumor efficacy and mechanisms of action of anti TRAIL-R2 mAb.

2. I am a co-inventor of U.S. application serial No. 10/721,763, "*Anti-Trail-R Antibody*," filed on May 17, 2002. I would get a predefined amount of remuneration from Kirin Pharma Company when the present application is granted. Further, it is expected that I would be remunerated when Kirin Pharma Company gains an income in commercializing a product or technology that is related to the invention of the present application.

3. I have read the U.S. Patent & Trademark Office's Office Action dated September 27, 2007, and publications cited there: Griffith *et al.*, *J. Immunol.* 162:2597 (1999); U.S. Patent No. 6,342,369; and U.S. 2003/0190687. I understand that Examiner Kaufman believes that certain prior-art antibodies, M413, 16E2, and TRA-8, can induce apoptosis when they are in monomeric form.

4. We conducted experiments on 16E2, and TRA-8 and concluded that none of these antibodies are a monoclonal antibody or antibody fragment that binds to TRAIL-R2 to induce apoptosis in carcinoma cells in a monomeric form. Rather, the studied antibodies only induced apoptosis when they were aggregated into a polymeric complex. The following paragraphs relate the results of the experiments in question, which concerned16E2, and TRA-8.

5. **16E2**: Examiner Kaufman likewise believes, I understand, that the 16E2 antibody induces apoptosis in carcinoma cells (SK-MES-1) when it is not cross-linked. The 16E2 antibody is a single-chain Fv (scFv), as described in U.S. Patent No. 6,342,369 (column 11, lines 41 to 55). Generally, scFv forms a polymer without cross-linking, as demonstrated, for instance, by Kortt *et al.*, *Protein Engineering* 10: 423, 1997 (appended). In particular, Kortt showed by gel filtration that affinity purified scFv antbodies yielded not only monomeric antibodies but also dimers and trimers. It should be notable that affinity purified scFv antibodies contained the approximate half amount of polymers (see Kortt's Figures 1 and 5).

6. We conducted experiments that demonstrated that a preparation of 16E2 antibodies contained a extremely large amount of polymeric forms of the antibody. More specifically, I produced the 16E2 antibody according to the method disclosed in U.S. Patent No. 6,342,369, and I then examined whether that antibody formed a polymer. Exhibit A to this declaration shows that almost all of the 16E2 antibodies formed dimeric and tetrameric polymers. I detected very little amount of monomeric 16E2, if any. Accordingly, I conclude that the apoptosis-inducing effects of the 16E2 antibody are due to its polymeric form; hence, that 16E2 does not function as "a single substance without polymer."

7. **TRA-8**: I understand Examiner Kaufman also to say that the TRA-8 antibody, when it is not cross-linked, induces apoptosis in Jurkat leukemia cells. Pursuant to testing detailed in Exhibit B to this declaration, however, I determined that the monomeric TRA-8 antibody did not induce apoptosis without cross-linking. More specifically, I found that the TRA-8 antibody had no apoptotic activity when it existed in culture medium but that TRA-8 could induce apoptosis only when it was cross-linked (see "Experiment 1" of Exhibit B). In

addition, I demonstrated that purified TRA-8 antibody contained both antibody monomer and polymer (see "Experiment 2"). I also found that the purified TRA-8 antibody had apoptosis-inducing activity without any cross-linker (see "Experiment 3"). Furthermore, I isolated the TRA-8 antibody monomer ("Experiment 4") and found that the antibody monomer did not have any apoptosis-inducing activity ("Experiment 5"). I conclude, therefore, that the TRA-8 antibody monomer does not induce apoptosis but that only the polymeric form of TRA-8 induces apoptosis in the absence of any cross-linker.

We also evaluate Griffith's M413 antibody as follows:

8. **M413**: Examiner Kaufman believes, I understand, that Griffith's M413 antibody induces apoptosis in carcinoma cells when it is not cross-linked, *i.e.*, when it is not "polymeric." Even without cross-linking, however, antibodies themselves generally form an aggregate spontaneously. For instance, we had demonstrated in Example 27 of the application that a purified antibody preparation contained both monomeric and polymeric forms of the antibody. See also our Example 28, which showed that the monomeric "0304" and "0322" antibodies fractionated by gel filtration chromatography exhibited cell-death-inducing activity against Colo205 cells without any cross-linker, but that the monomeric "H-48-2" antibody had no cell-death inducing activity on Colo205 cells. Thus, the polymeric form of H-48-2 antibody only exhibited cell-death-inducing activity in the absence of any cross-linkers. For that reason it is possible that any apoptosis reported by Griffith was due to a naturally-aggregated polymeric fraction of the M413 antibody preparation, and not to a 100% preparation of a monomeric form of the antibody. We could not specifically test M413 antibody because it is not available to the public; that is, the M413 antibody is not commercially available and has not been placed in a depository institution. Accordingly, it was impossible for us to evaluate the structural and functional characteristics of M413.

9. In summary, I conclude that none of the prior-art antibodies can induce apoptosis in monomeric form. We found that the prior-art antibodies were able to induce apoptosis only when they formed polymeric antibody aggregates.

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I hereby declare that all the statements made herein of my known knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United

States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

DATE

KAZUHIRO MOTOKI

EXHIBIT A

Exhibit A

Construction of 16E2 scFv with 6xHis tag

The nucleotide coding 16E2 scFv with 6xHis tag, described in SEQ ID NO:6 and Figure 15A of WO98/51793, was synthesized by PCR. For ease of cloning into expression vector, NdeI and SalI sites were inserted in 5 prime and 3 prime of PCR-amplified fragment respectively. The fragment derived from PCR was cloned into pCR4Blunt-TOPO (Invitrogen) for the sequence confirmation. After the pCR4Blunt-TOPO with the inserted PCR fragment was treated with NdeI and SalI, the resultant fragment was sub-cloned into pET32b(+) (Novagen). Hereinafter this constructed vector is described as "pET32b(+)-16E2 scFv with 6xHis tag".

Purification of 16E2 scFv

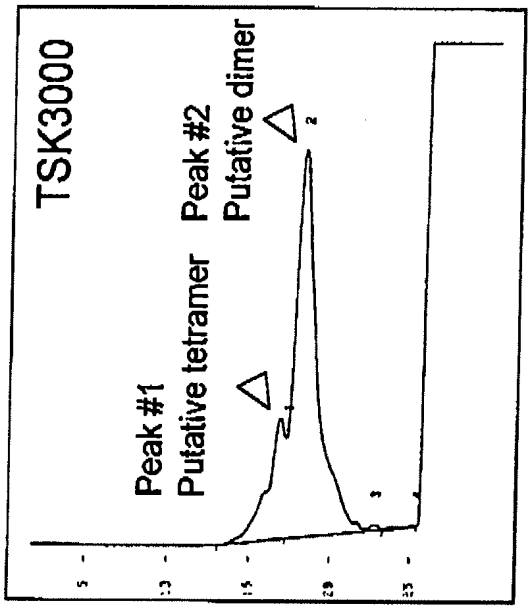
For protein purification of 16E2 scFv, E.coli strain Rosetta™ (DE3) Competent Cells (Novagen) was transformed with pET32b (+)-16E2 scFv with 6xHis tag. Transformed E.coli was cultured in 20 mL of LB medium with ampicillin (50 µg/mL) overnight at 37 °C. 5 mL of cultured E.coli were diluted into 500 mL of MagicMediaTM E.coli Expression Medium (Invitrogen). To induce expression of 16E2 scFv, diluted E.coli was cultured overnight at 30 °C. After the value of OD600 was measured, cells were harvested by centrifugation (4000xg for 5 min). To extract the periplasmic fraction, 1 mL of periplasmic extraction buffer (100 mM Tris-HCL, 20 mM EDTA, pH7.4) was used to resuspend per 9 OD600 units of cell pellets. 372 mL of resuspended cells were incubated at 30 °C overnight. Cell debris was removed by centrifugation. The supernatants were concentrated up to about 130 mL with Vivaspin 20 mL (Sartorius) and dialyzed into lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM Imidazole, pH8.0). Ni-NTA agarose resin (Qiagen) was used according to the manufacturer's instructions. The dialyzed supernatants were added to the resin equilibrated with lysis buffer in a gravity flow column. The resin was then washed with wash buffer (50 mM NaH2PO4, 300 mM NaCl, 60 mM Imidazole, pH8.0). Proteins were eluted with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM Imidazole, pH8.0). Excess salt and imidazole was removed by dialysis with PBS. Concentration was estimated as A280 nm of 1.65 = 1 mg/mL.

Analysis of purified 16E2 scFv with size exclusion chromatography

20 µg of purified 16E2 scFv was applied to TSK G3000 SWXL size exclusion column (7.8 x 300 mm, Tosoh) with a mobile phase of 20 mM sodium phosphate, pH 7.0, plus 500 mM NaCl at a flow rate of 0.5 mL/min. Chromatograms were obtained by monitoring absorbance at 215 nm. Most of purified 16E2 scFv existed as putative dimeric and tetrameric forms (Figure1) compared with a set of molecular weight standard marker (Oriental Yeast). Little monomeric form was observed (Figure 1). Multi angle laser light scattering analysis with TSK G2000 SW size exclusion column (Tosoh) revealed the molecular weights of the peaks, indicating that major two peaks were dimer and tetramer respectively (data not shown).

Exhibit A

Figure 1



Peak No.	1	2
Retention time (min)	16.885	18.14
MW (kDa)	96.91672	53.7316

EXHIBIT B

Exhibit B

Experiment 1 Preparation of a culture supernatant of TRA-8 hybridoma and measurements of an antibody concentration and the activity of inducing apoptosis of the culture supernatant

Materials and Methods

Hybridoma TRA-8 which was internationally deposited was obtained and cultured in DMEM medium containing 50units/ml penicillin, 50μg/ml streptomycin, glutamine, IL-6 and 10% FCS (SIGMA). The supernatant was collected, and the antibody concentration in the supernatant was measured by ELISA using anti mouse IgG antibody (Sigma) and horseradish conjugated goat anti-mouse IgG (γ) antibody.

The activity of inducing apoptosis against Colo225 colon cancer (ATCC NO.CCL-222) of the supernatant of the hybridoma which produces mouse anti-TRAIL-R2 monoclonal antibody was measured. Colo205 cells cultured in RPMI medium containing 10% FCS were adjusted to $1x10^4$ or $1x10^5$ cells/ml and added into 96 well flat bottom plate at 100μl/well. Then, hybridoma culture supernatant was added at 10μl/well to the wells such that final antibody concentration became 1, 10, 100 or 1000ng/ml. Wells (cross-linking agent +) were added with 10μl of goat anti-mouse IgG(γ) specific polyclonal antibody (SIGMA) as a cross-linking agent at a final concentration of 10μg/well and wells (cross-linking agent -) were added with 10μl of RPMI medium. A well for a negative control was added with the hybridoma culture supernatant. Cells were cultured for 48 hours at 37℃ under 5% CO_2 and 20μl of MTS reagent (CellTiter 96 AQUEOUS Non-Radioactive Cell Proliferation Assay; PROMEGA) which was prepared according to the manufacturer's instruction was added to each well. After cultured for 1 to 3 hours at 37℃ under 5% CO_2, absorbance at 490nm (reference wave length is 630nm) was measured using a micro plate reader (1420 ARVO multi label counter; WALLAC). Cell viability was calculated using the reducing ability of mitochondria as an index. The formula to calculate the cell viability was as follows.

Cell viability (%) = 100x(a-b)/(c-b) [where, a is the measured value of well in which cells were added, b is the measured value of cell-free wells, c is the measured value of the control well.]

Results

Culture supernatant of TRA-8 hybridoma did not induce apoptosis at all against 10000cells/well of Colo205 in the absence of the cross-linking agent (Figure 1B). When

a number of cells was reduced to 1000cells/well such that apoptosis was prone to be induced, it did not induce apoptosis at all in the absence of the cross-linking agent (figure 1A). On the contrary, culture supernatant of TRA-8 hybridoma induced apoptosis dependent on the concentration against Colo 205 (Figures 1C and 1D) in the presence of the cross-linking agent.

Experiment 2 Antibody preparation from a hybridoma and the analysis of monomer and polymer (aggregate) content

Materials and Methods

Hybridoma TRA-8 was conditioned in eRDF medium (KYOKUTO PHARMACEUTICAL INDUSTRIAL CO.,LTD) containing 10ng/ml Recombinant Human IL-6 (R&D Systems), 10% Low IgG Fetal Bovine Serum (HyClone) and freezed. A portion of the hybridoma was conditioned in eRDF medium containing 1 x Insulin-Transferrin-Seleniun-X Supplement (GIBCO BR), 10ng/ml Recombinant Human IL-6 (R&D Systems) and 1 % Low IgG Fetal Bovine Serum (HyClone). The hybridoma was cultured in a flask and culture supernatant was collected when the viability of the hybridoma reached 90%. The collected supernatant was filtered with 10μm filter and 0.2μm filter (GERMAN SCIENCE) to delete impurities.

An antibody was affinity-purified from culture supernatant containing antibody using a column (0.8x40cm) containing Protein A (Amersham Pharmacia Biotech). PBS was used as an absorption buffer and Glycine buffer (pH3) was used as an elution buffer. The eluted fraction was adjusted to about pH7.2 with 1M Tris (pH9.0). The buffer of the purified antibody was replaced with PBS using a dialysis membrane (10000 cut, Spectrum Laboratories) and sterilized by filtration using 0.22μm membrane filter MILLEX-GV (MILLIPORE) to obtain a purified antibody. The concentration of the purified antibody was determined by measuring the absorption at 280nm provided that 1.4OD corresponds to 1mg/ml antibody. Monomer and polymer content of the purified antibody was measured using HPLC (SHIMADZU CORPORATION, LC-07) and TSKgel-G3000SWxL (TOSO) as a column. The absorption of the eluates was output to a recorder and monomer and polymer content was calculated by peak areas of the monomer and polymer.

Results

Table 1 shows the amount of the purified antibody and the monomer and polymer content. The purified TRA-8 included the polymer at relatively high ratio.

Table 1 Antibody content and monomer/polymer content (%) of TRA-8

Antibody	Antibody conc.	Antibody amount	Monomer/polymer content
TRA-8	1.85 mg/mL	5.55 mg	95.7% / 4.26 %

Experiment 3 Apoptosis inducing activity of the purified TRA-8

The apoptosis inducing activity against colon cancer cell line Colo 205 (ATCC No.CCL-222) of the purified TRA-8 was measured. Colo 205 cells which were cultured in RPMI medium containing 10% FCS was adjusted to 1.0 x 10^5 cells/ml and 100µl was added to wells of 96 well flat bottom plate (Becton Dickinson). The cells were cultured overnight at 37℃ under 5% CO_2. Then, the purified TRA-8 antibody (10µl/well) was added to each well at a final concentration of 1, 3, 10, 30, 100, 300 and 1000ng/ml. Wells (cross-linking agent +) were added with 10µl of goat anti-mouse IgG(γ) specific polyclonal antibody (Sigma) as a cross-linking agent at a final concentration of 10µg/well and wells (cross-linking agent -) were added with 10µl of RPMI medium. A purified human anti DNP antibody (IgG1) was used as a negative control.

Cells were cultured for 48hours at 37℃ under 5% CO_2 to react the antibody to a receptor on the cell surface. Then, 20µl of MTS reagent (CellTiter 96 AQUEOUS Non-Radioactive Cell Proliferation Assay; Promega) which was prepared according to the manufacturer's instruction was added to each well. After cultured for 1 to 3 hours at 37℃ under 5% CO_2, absorbance at 490nm (reference wave length is 630nm) was measured using a micro plate reader (1420 ARVO multi label counter; WALLAC). Cell viability was calculated using the reducing ability of mitochondria as an index. The formula to calculate the cell viability was the same with that of Experiment 1.

Results

Figure 2 shows the results. The purified TRA-8 induced apoptosis in the absence of the cross-linking agent dependent on the concentration.

Experiment 4 Fractionation of TRA-8 monomer

TRA-8 obtained by Experiment 3 above was fractionated using HPLC (L-6020/LC-6320, Hitachi). Antibody was loaded on TSKgel-G3000SWxL column (TOSO) equilibrated with PBS at 0.5ml/min. of flow rate. A fraction collector (FC205, GILSON) was used to collect the eluates at a volume of 0.5ml/fraction. The collected fractions were sterilized

using 0.22μm membrane filter, ULTRAFREE-MC STERILE (MILLIPORE). Then, the absorption at 280nm was measured to calculate the antibody concentration of each fraction. The concentration was calculated provided that 1.4OD corresponds to 1mg/ml. The fractions around monomer peak were subjected to HPLC to be analyzed monomer and polymer content.

Results

The amounts of the antibody contained in the monomer fraction of TRA-8 and the monomer and polymer content were shown in Table 2.

Fractions #14 and #15 mainly contained the antibody. Fraction #15 further contained substances which were deduced to be degradates. The polymer was not detected in Fraction #15. Thus obtained fractions were thought to be a highly purified monomer fraction.

Table 2 Monomer purity and polymer content of TRA-8

mAb	fraction #	A280(x0.25)	A280(x1)	Conc. (mg/ml)	Polymer(%)	Monomer(%)	Degradates(%)
TRA-8	13	0	0.008	0.000	-	-	-
	14	0.2528	1.011	0.722	0.3043	99.0016	0.6941
	15	0.1813	0.725	0.518	0	99.5637	0.4363
	16	0.0192	0.077	0.055	0	98.0724	1.9276

*Sample apply: 2mg/ml 420-440 ul

**Conc. = A280(x1)/1.4

Experiment 5 Apoptosis inducing activity

Materials and Methods

The activity of inducing apoptosis against Colo205 cells (ATCC No.CCL-222) of the highly purified monomer fraction was detected. Colo 205 cells which were cultured in RPMI medium containing 10% FCS was adjusted to 1.0 x 10^5 cells/ml and 100μl was added to wells of 96 well flat bottom plate (Becton Dickinson). The cells were cultured for 24hours at 37℃ under 5% CO_2. Then, the fractionated TRA-8 antibody (10μl/well) was added to each well at a final concentration of 1, 3, 10, 30, 100, 300 and 1000ng/ml. Cells were cultured for 48hours at 37℃ under 5% CO_2 to react the antibody to a receptor on the cell surface. Then, 20μl of MTS reagent (CellTiter 96 AQUEOUS Non-Radioactive Cell Proliferation Assay; Promega) which was prepared according to the manufacturer's instruction was added to each well. After cultured for 2 hours at 37℃ under 5% CO_2, absorbance at 490nm (reference wave length is 630nm) was

measured using a micro plate reader (1420 ARVO multi label counter; WALLAC). Cell viability was calculated using the reducing ability of mitochondria as an index. The formula to calculate the cell viability was the same with that of Experiment 1.

Results

Figure 3 shows the results. The monomer fraction of TRA-8 did not induce apoptosis against Colo205 at all. Therefore, it was revealed that the activity of inducing TRA-8 in the absence of the cross-linking agent depended on the polymer.

Figure 1

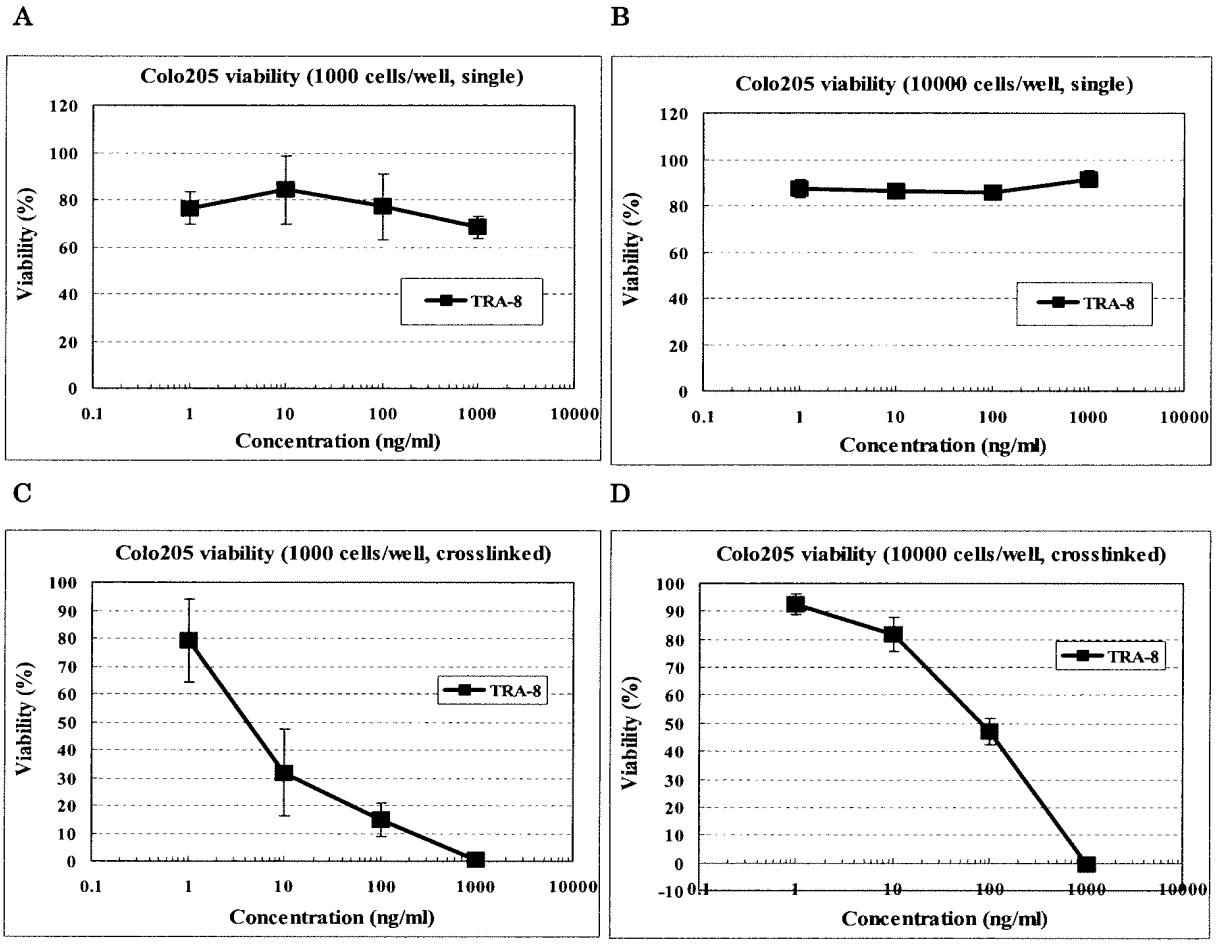


Figure 1 Activity of inducing apoptosis against Colo205 of TRA-8

Figure 2

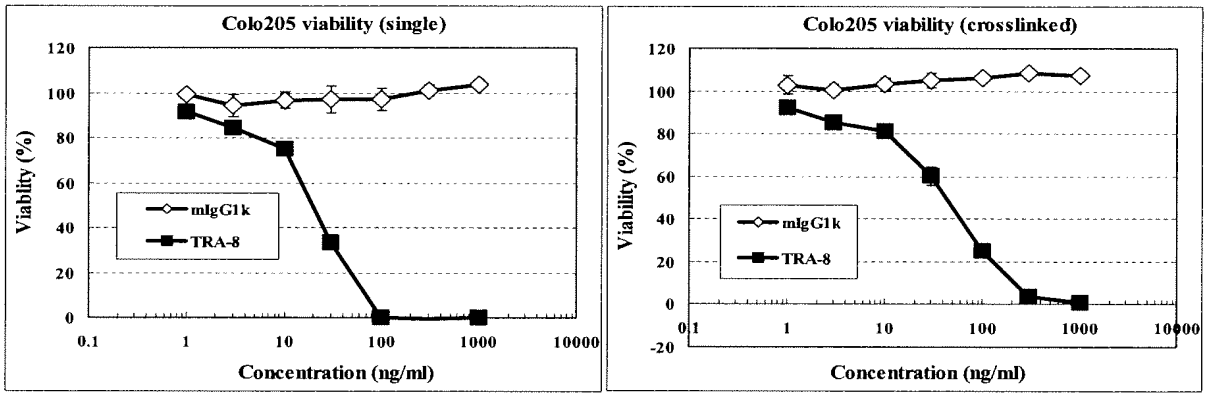


Figure 2 Activity of inducing apoptosis against Colo205 of the purified TRA-8

Figure 3

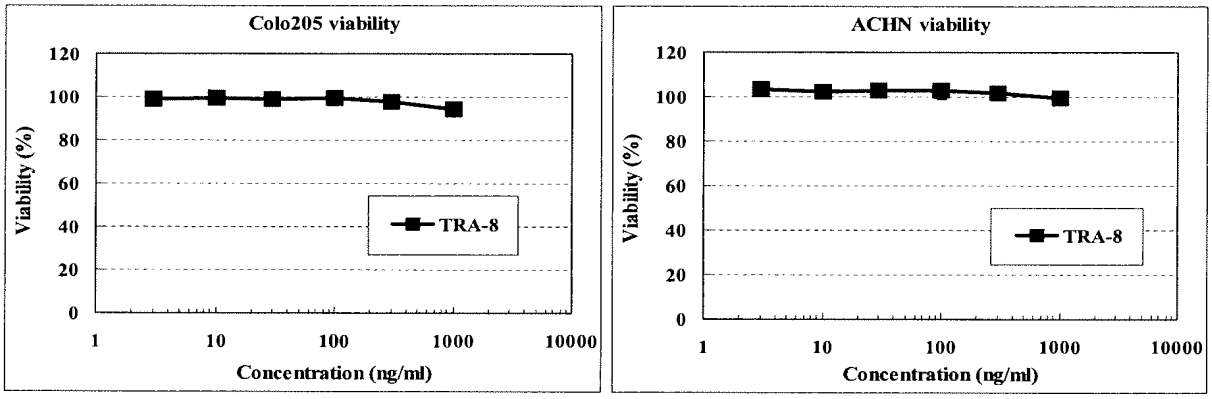


Figure 3 Activity of inducing apoptosis against Colo205 of the highly purified monomer fraction of TRA-8